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Published in:
Accounts of Chemical Research

DOI:
10.1021/acs.accounts.9b00177

Publication date:
2019

ACCOUNTS of chemical research

Article

Cite This: Acc. Chem. Res. XXXX, XXX, XXX−XXX

pubs.acs.org/accounts

Carriers Break Barriers in Drug Delivery: Endocytosis and Endosomal Escape of Gene Delivery Vectors

Published as part of the Accounts of Chemical Research special issue "Nanomedicine and Beyond".

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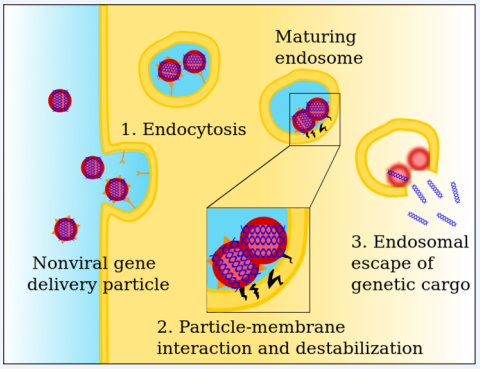
CONSPECTUS: Over the past decades, major efforts were undertaken to develop devices on a nanoscale level for the efficient and nontoxic delivery of molecules to tissues and cells, for the purpose of either diagnosis or treatment of disease. The application of such devices in drug delivery has proven to be beneficial for matters as diverse as drug solubility, drug targeting, controlled drug release, and transport of drugs across cellular barriers. Multiple nanotherapeutics have been approved for clinical treatment, and more products are being evaluated in preclinical and clinical trials. However, many biological barriers hinder the medical application of nanocarriers. There are two main classes of barriers that need to be overcome by drug nanocarriers: extracellular and intracellular barriers, both of which may capture and/or destroy therapeutics before they reach their target site. This Account discusses major biological barriers that are confronted by nanotherapeutics, following their systemic administration, focusing on cellular entry and endosomal escape of gene delivery vectors. The use of pH-responsive materials to overcome the endosomal barrier is addressed.



Historically, cell biologists have studied the interaction between cells and pathogens in order to unveil the mechanisms of endocytosis and cell signaling. Meanwhile, it is becoming clear that cells may respond in similar ways to artificial drug delivery systems and, consequently, that knowledge on the cellular response against both pathogens and nanoparticulate systems will aid in the design of improved nanomedicine. A close collaboration between bioengineers and cell biologists will promote this development. At the same time, we have come to realize that tools that we use to study fundamental cellular processes, including metabolic inhibitors of endocytosis and overexpression/downregulation of proteins, may cause changes in cellular physiology. This calls for the implementation of refined methods to study nanocarrier−cell interactions, as is discussed in this Account.

Finally, recent papers on the dynamics of cargo release from endosomes by means of live cell imaging have significantly advanced our understanding of the transfection process. They have initiated discussion (among others) on the limited number of endosomal escape events in transfection, and on the endosomal stage at which genetic cargo is most efficiently released. Advancements in imaging techniques, including super-resolution microscopy, in concert with techniques to label endogenous proteins and/or label proteins with synthetic fluorophores, will contribute to a more detailed understanding of nanocarrier-cell dynamics, which is imperative for the development of safe and efficient nanomedicine.

1. INTRODUCTION

Overcoming biological barriers remains a major challenge in the effective delivery of therapeutic agents to diseased sites. Indeed, such barriers exist in the process of accomplishing an appropriate biodistribution, but also in cellular uptake and intracellular routing. Approaches to overcome these barriers have received significant attention over the past decades. Among others, advances in nanotechnology have improved the delivery of therapeutics via nanosized carriers to desired tissues and cells. In the field of gene delivery, nanoparticles have been translated to the clinic as a promising platform. However, most of the nanocarriers, belonging to the class of nonviral vectors, are still primarily in a developing, preclinical stage because of their relative delivery inefficiency, when compared to viral vectors.[1,2] Apart from being confronted with various extracellular hurdles, additional barriers arise for nanocarriers when they encounter the target cells. Effective internalization

Received: April 10, 2019

DOI: 10.1021/acs.accounts.9b00177
Acc. Chem. Res. XXXX, XXX, XXX−XXX

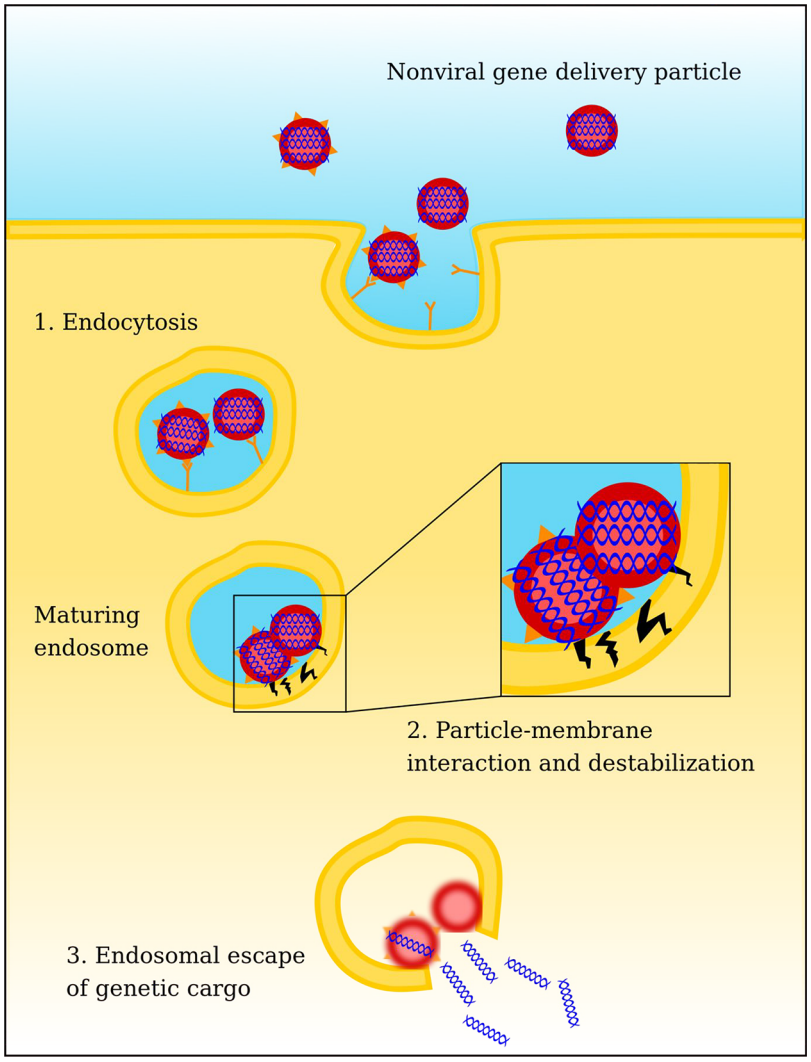


Figure 1. Endocytosis of gene delivery particles. (1) Binding of gene delivery particles to the cell surface, through electrostatic interactions between the positively charged particles and the negatively charged cell surface and/or ligand−receptor interaction, results in their endocytosis. (2) Interaction of the particles with the endosome causes destabilization of the particles and the endosomal membrane. (3) Genetic cargo that has dissociated from the particle is released into the cytosol through the endosomal membrane destabilization.

and subsequent release of their cargo, requiring translocation across endosomal and/or nuclear membranes, constitute an additional parameter in determining therapeutic efficiency, and hence, potential clinical impact. Therefore, special properties are required for nanocarriers to cope with refractory extra- and intracellular conditions, both in vivo and in vitro, which also include issues of (transient) stability and low cytotoxicity. A better understanding of nanocarrier−cell interactions will help to increase the efficacy, safety, and clinical translation of nanocarriers.

2. CELLULAR UPTAKE OF GENE DELIVERY VECTORS

Gene delivery with nonviral vectors, including lipoplexes and polyplexes, still suffers from limited efficiency compared to viral gene delivery. To optimize nonviral gene delivery, a detailed understanding of the mechanisms through which the genetic cargo is delivered into a cell is vital. In 1995, Wrobel and Collins showed that lipoplexes, i.e., complexes between cationic lipids and nucleic acids, do not fuse with the plasma membrane of cells to deliver their genetic cargo into the cytoplasm, but become internalized via the process of endocytosis.[3] In order to achieve a therapeutic effect, the nucleic acids need to reach the desired cell compartment, i.e., the nucleus for DNA or the cytosol for RNA. As a result, the endosomal membrane constitutes a barrier for the release of genetic cargo from endocytosed gene vectors into the cytosol (Figure 1).

2.1. Endocytosis of Gene Delivery Vectors

Typically, endocytosis is subdivided in pinocytosis and phagocytosis, where pinocytosis includes clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, fluid phase endocytosis, and macropinocytosis. How these different pathways are regulated is largely unclear, and is dependent on cell type and cell state.[4]

By the use of biochemical and genetic approaches to inhibit endocytosis, and monitoring simultaneously clathrin-mediated endocytosis of transferrin, Zuhorn et al. showed that CME is the major entry pathway for SAINT-2/DOPE lipoplexes in COS-7 cells.[5] This was the first report in which a specific type of endocytosis, namely, CME, was linked to productive transfection with lipoplexes. Later, branched PEI (BPEI) polyplexes were shown to enter HeLa cells via multiple

DOI: 10.1021/acs.accounts.9b00177
Acc. Chem. Res. XXXX, XXX, XXX−XXX

endocytic pathways, including CME, macropinocytosis, and caveolae.[6−8] (Nontargeted) lipoplexes and polyplexes have been shown to bind to cells via adhesion receptors, including integrins and syndecans.[9−12] To improve delivery to specific cell types, functionalization of the nanoparticle surface with ligands that target cell-specific receptors is successfully used in vitro as well as in vivo.[13−16]

2.2. Refined Methods to Study Endocytosis

Chemical inhibitors of endocytosis are widely used to study the involvement of specific uptake routes. They act quickly, and their action is often reversible. However, chemical inhibitors of endocytosis almost never completely nor specifically block one endocytic pathway, and may induce cellular toxicity.[17] For example, Pitstop 2, a clathrin inhibitor, was shown to also inhibit clathrin-independent endocytosis.[18] Therefore, the effect of an endocytosis inhibitor on the uptake of a specific nanocarrier should be compared to its effect on the uptake of reference substances.[19,20] Also, colocalization of a nanocarrier with reference substances may be helpful in determining its route of entry. Transferrin is a bona fide cargo for the clathrin-mediated endocytosis pathway. However, the possibility of direct binding of transferrin to the nanoparticle under investigation, which could result in false-positive colocalization, should be excluded. In addition, one should take into account the difference in kinetics between transferrin and nanocarrier uptake. Typically, transferrin uptake reaches near maximum levels after 10 min of incubation,[21] while nanoparticles (∼100 nm) reach maximum uptake after several hours. Importantly, colocalization of nanoparticles with clathrin, especially after prolonged incubation times, is not a proof for the involvement of clathrin-mediated endocytosis, because clathrin is also involved in the pinching off of vesicles from sorting endosomes, i.e., in the further processing of endosomes that occurs *after* the internalization event at the plasma membrane. Cholera toxin B is often used as a marker cargo for caveolar endocytosis, but can also be internalized via routes that do not involve caveolae.[22,23] Since caveolae seem to be instrumental in the uptake of excess glycosphingolipids, the glycosphingolipid lactosylceramide (LacCer) may be a better marker for caveolar endocytosis.[24]

Next to the use of marker cargoes for specific routes of endocytosis, colocalization studies by means of overexpression of fluorescent fusion proteins of key endocytic regulators or immunostaining for these regulators can be helpful in determining routes of uptake for nanocarriers. In addition, downregulation of endocytic proteins and overexpression of dominant negative forms is being used to pinpoint the involvement of specific routes of uptake. However, overexpression of wildtype and dominant negative forms of proteins, by means of plasmid constructs that are typically under the control of strong promotors such as the CMV promotor, may cause artifacts.[25,26] For example, overexpression of caveolin-GFP may result in its accumulation in endolysosomes without prior localization in caveolae, which has been mistaken for localization in caveolae-positive endosomes.[27] The CRISPR/Cas9 genome editing technique allows for the expression of genes under the control of their natural promotor, preventing cellular defects that may occur due to protein overexpression. Shvets et al. used CRIPSR/Cas9 to generate double genome-edited cells, expressing caveolin1-GFP and cavin 1-mCherry.[28] Because both caveolin and cavin proteins are needed to form caveolae, the combined presence of caveolin and cavin in vesicles, reveals the presence of intact caveolar vesicles. The fluorescent tagging of endogenous endocytic proteins by means of CRISPR/Cas9 permits the visualization of endocytosis under physiological conditions. However, this method is limited to genetically encoded fluorophores, i.e., fluorescent proteins. This may be disadvantageous because a "bulky" fluorescent tag may hinder interactions of the fluorescently tagged protein with cellular components, thereby disturbing its physiological function.

In order to label intracellular proteins with (smaller) synthetic fluorophores, cells can be genetically encoded with SNAP-tag, and -subsequently- be incubated with membrane-permeable benzylguanine-fluorophores, leading to the formation of a covalent bond between the SNAP-tag and the benzylguanine-fluorophore.[29] Introduction of a disulfide bond in between the benzylguanine group and the fluorophore gives the possibility to cleave the fluorophore from the protein by means of a reducing agent. A cell-impermeable reducing agent can then be used to cleave the label that is present extracellularly, permitting specific labeling of the intracellular pool of SNAP-tagged protein. Bitsikas et al. used a GPI-anchored SNAP-tag construct to detect internalized GPI-AP with very high efficiency and low background.[30] Moreover, spectroscopic properties of synthetic fluorophores are often better than that of fluorescent proteins, which is especially important for applications in live cell imaging. In the same work, Bitsikas et al. used biotinylation of the entire pool of plasma membrane proteins to define all primary endocytic vesicles. They showed that ∼95% of endocytic vesicles originated from clathrin-mediated endocytosis. Moreover, they showed that CME is the main pathway for uptake of GPI-anchored proteins, which is commonly believed to occur via the CLIC/GEEC pathway.[31] Inhibition of CME, through overexpression of C-terminal AP180 (AP180/C) or dominant-negative dynamin (K44A), led to a reduction in uptake of the GPI-anchored protein CD59. Strikingly, in cells overexpressing an AP2 mutant deficient in binding the transferrin receptor, transferrin uptake was significantly reduced, whereas the uptake of CD59 was significantly increased. Since GPI-anchored proteins lack the cytosolic domains that are typically recognized by adaptor proteins to recruit receptors to coated pits in receptor-mediated endocytosis, they reasoned that GPI-APs are normally excluded from coated pits by steric crowding effects. These data suggest that elimination of adaptor protein-mediated recruitment of receptors to coated pits alleviates the steric exclusion of GPI-anchored proteins. Thus, perturbations of coated pit formation may have a more severe impact on high affinity proteins, such as the transferrin receptor, than low affinity proteins, such as CD59.[30]

Taking into account the perturbations of physiological processes that may be induced by the commonly used methods to study endocytosis, a thorough (re)investigation of the cellular trafficking pathways of gene delivery vectors by means of the aforementioned refined methods, including the execution of proper controls, is warranted. Paradoxically, transfection of cells (by means of gene delivery vectors) is a method that is widely used in the study of endocytosis, and may cause significant (temporary) changes in endocytic processes. For example, transfection of cells with PEI polyplexes was shown to cause a reduction in the number of early endosomes.[32] Moreover, transfection with both lipoplexes and polyplexes has been linked to the induction of autophagy.[33]

DOI: 10.1021/acs.accounts.9b00177
Acc. Chem. Res. XXXX, XXX, XXX−XXX

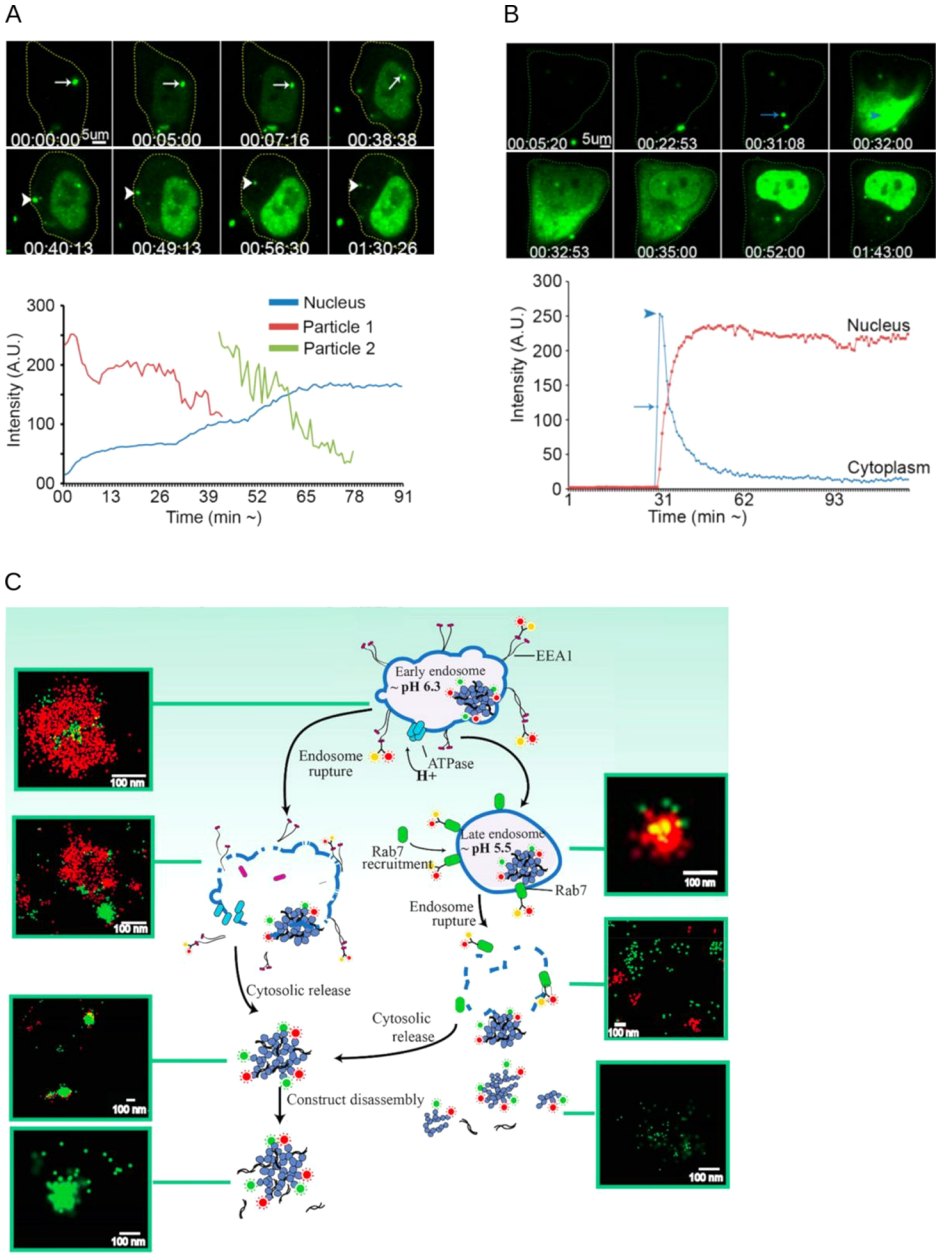


Figure 2. Endosomal escape mediated by gene delivery particles. (A) In lipoplex-mediated gene delivery, ODN (green) release from multiple endosomes results in gradual accumulation of ODNs in the nucleus. (B) In polyplex-mediated gene delivery, a burst release of the genetic cargo from typically only one or two endosomes per cell, with a complete discharge of the nucleic acid content from the endosome into the cytosol, results in instantaneous accumulation of ODNs in the nucleus. (A, B) Graphs show the fluorescence intensity of particles, cytoplasm and nucleus over time. Adapted with permission from ref 49. Copyright 2013 American Chemical Society. (C) Glycoplexes (green) are initially present in early and late endosomes (red). They are released intact in the cytosol where they disassemble. Adapted with permission from ref 57. Copyright 2018 American Chemical Society.

3. INTRACELLULAR PROCESSING OF GENE DELIVERY DEVICES

The processing of endosomes within the various uptake pathways differs and may consequently influence opportunities for endosomal escape and release of cargo into the cytoplasm, as mediated by gene vectors. Especially, because the mechanisms of endosomal escape differ between different types of gene vectors. For instance, the endosomal escape of lipoplexes involves lipid mixing between cationic lipids in the lipoplex with anionic lipids in the endosomal membrane, and is pH-independent.[34] On the contrary, the endosomal escape of polyplexes, i.e., complexes between cationic protonatable polymers and nucleic acids, is dependent on endosomal acidification. Specifically, the endosomal escape of polyplexes has been suggested to result from their H+ buffering capacity resulting in enhanced Cl- accumulation within the endosome and eventually osmotic lysis of the endosomal membrane (proton sponge effect).[35] While branched PEI (BPEI) polyplexes were shown to enter HeLa cells via CME, macropinocytosis, and caveolae, only the uptake of polyplexes via caveolae resulted in productive transfection.[8] Transfection by polyplexes via a pH neutral caveolar pathway would not be supportive for the fact that polyplex-mediated endosomal escape of genetic cargo is dependent on endosomal acid-

DOI: 10.1021/acs.accounts.9b00177
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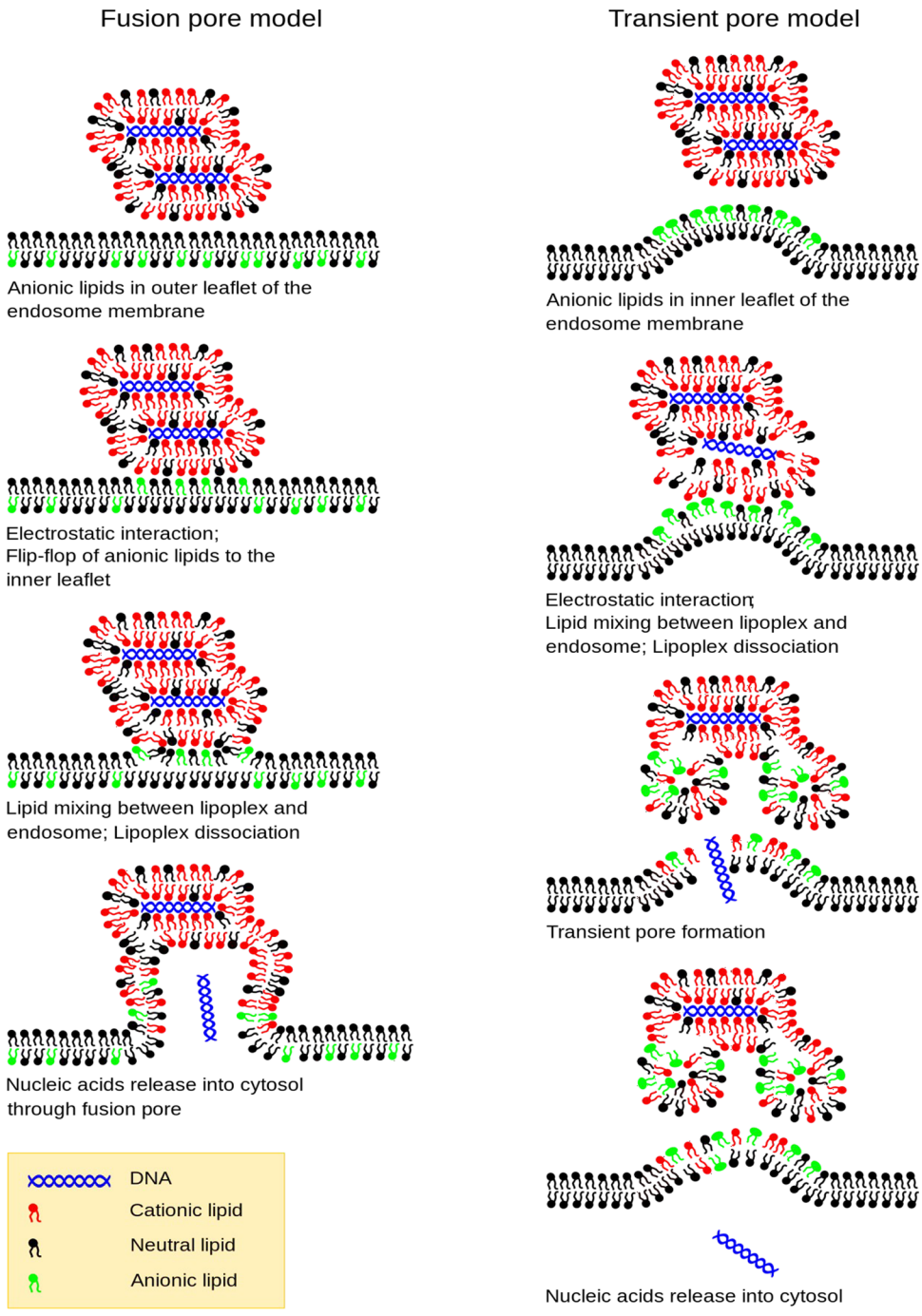

Figure 3. Old and new model for cationic lipid-mediated endosomal escape of genetic cargo. The fusion pore model (left column) proposes that nucleic acid release into cytosol occurs through fusion of the lipoplex with the endosome membrane, initiated by flip-flop of anionic PS lipids from the outer leaflet of the endosome to the inner leaflet. The transient pore model (right column) proposes that nucleic acid release into cytosol occurs via transient pores in the endosome membrane. LBPA in the inner leaflet of the endosome membrane may play a role in membrane permeabilization.

ification, i.e., the proton sponge theory. However, it has been shown that endosomes from distinct endocytic pathways may merge, which would allow for acidification of vesicles originally derived from caveolae.[36]

Remarkably, when polyplex-containing endosomes in the CME pathway were halted, by means of PKA inhibition, at a stage between early and late endosomes, they contributed to the overall transfection.[8] Specifically, localization of BPEI polyplexes in transferrin$^-$/rab9$^-$/LAMP1$^-$ endosomal compartments resulted in effective endosomal escape of genetic cargo.[8] Probably, polyplex-mediated endosomal swelling and/or polyplex dissociation within the CME pathway is too slow to induce endosomal escape prior to polyplex accumulation in degradative lysosomal compartments. Therefore, "freezing" the endosomal processing, by means of PKA inhibition, at a prelysosomal stage broadens the window of opportunity for release of genetic cargo from polyplex-containing endosomes within the CME pathway, thereby enhancing transfection efficiency. A similar intracellular compartment, i.e., maturing endosomes, was found as the site for endosomal escape of

DOI: 10.1021/acs.accounts.9b00177
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siRNA in lipoplex- and LNP-mediated delivery.[37,38] Apparently, irrespective of the different mechanisms that are involved in endosomal escape mediated by lipoplexes and polyplexes, the compartment that allows for their effective escape seems to be the same, which may suggest a common denominator in the two seemingly distinct mechanisms. The coformulation of drugs that halt gene vectors at a "release-prone" endosomal stage may be useful in the development of improved gene delivery vectors. Likewise, osmotic lysis of endosomes, for example via nigericin-mediated K+/H+ exchange,[39] may facilitate endosomal escape.

4. ENDOSOMAL ESCAPE OF GENE DELIVERY DEVICES

4.1. Lipoplex-Mediated Endosomal Escape

In the early days of nonviral gene delivery research, structure−function-activity relationships were primarily found by determining the physicochemical characteristics of lipoplexes and correlating those with transfection performance.[40−43] Major insight in the mechanism of lipoplex-mediated transfection was given by the investigation of lipid conformation by small-angle X-ray scattering (SAXS). Koltover et al. showed that an inverted hexagonal phase of lipoplexes, as opposed to a lamellar phase, stimulated DNA release from lipoplexes upon their incubation with anionic vesicles, which served as a mimic of the endosomal membrane.[44] They proposed a correlation between nonlamellar lipid organization in lipoplexes with transfection efficiency. The correlation between a nonbilayer phase of the lipoplex and its high transfection activity is still being reported in literature,[45,46] but does not seem to be a general finding. For example, Tassler et al. found that lysine-based amino-functionalized lipids, that varied in their chain length and (un)saturation, formed cubic or lamellar lipoplexes. The lamellar lipoplexes were the most efficient in transfection.[47] Logically, for efficient transfection, a lipoplex should induce nonbilayer phase structures upon interaction with anionic lipids at the endosome membrane but does not need to be in a nonbilayer conformation by itself. Indeed, Zuhorn et al. reported a correlation between the nonlamellar lipid phase of lipoplexes *after* incubation with anionic vesicles, and high in vitro transfection efficiency. This was supported by an efficient endosomal escape of genetic cargo, as measured by the nuclear accumulation of oligonucleotides (ODNs) in cells.[48] Overall, it is important to confirm the endosomolytic potential of nanocarriers, as determined by certain biophysical and/or structural characteristics, using cell-based assays to quantify the endosomal escape of nucleic acids.

Recently, by taking a live cell imaging approach to determine the intracellular processing of nonviral gene vectors, an important breakthrough in elucidation of the endosomal escape of nucleic acids was achieved. Specifically, incubation of HeLa cells with Lipofectamine2000 lipoplexes, comprising fluorescently labeled lipids and nucleic acid cargo (ODNs), revealed ODN release from multiple endosomes resulting in a gradual accumulation of ODNs in the nucleus (Figure 2A).[49] Release of genetic cargo from endosomes into the cytosol was suggested to occur via transient pores (Figure 3; right column),[49] and not through fusion of the lipoplex with the endosome membrane (Figure 3; left column).[34] Using a similar live cell imaging approach, Wittrup et al. showed the release of siRNA from endosomes in lipoplex- and LNP-mediated delivery. They observed between one and five release events per cell over several hours, with an incomplete release of genetic cargo from endosomes.[37]

The identification of a release-prone endocytic compartment, as discussed above, has initiated discussion on the involvement of a specific class of anionic lipids in mediating endosomal escape. Durymanov and Reineke questioned why lipoplexes release their genetic payload from maturing endosomes,[50] because the anionic phosphatidyl serine (PS) lipid content in maturing endosomes is less than in early endosomes,[51] while anionic PS at the cytoplasmic leaflet of the endosome has been held responsible for inducing endosomal escape (Figure 3; left column).[34] They suggest that LBPA at the limiting membrane of multivesicular bodies (MVBs) is the mostly likely candidate for inducing endosomal release of genetic cargo (Figure 3; right column). To our knowledge, no studies have (yet) been undertaken to investigate the lipid compositions of endosomes from different endocytic pathways and/or at different stages of maturation in relation to delivery efficiency by specific carrier systems. Recent advances in mass spectrometry for lipidomics make it feasible to embark on such studies.

4.2. Polyplex-Mediated Endosomal Escape

Polyplexes are considered to escape from endosomes through the proton sponge effect, an osmotically induced swelling of the endosome, triggered by the proton buffering capacity of the polyplexes, which results in rupturing of the endosomal membrane.[35] However, increasing evidence indicates that the proton sponge effect is not the only reason for polyplex-mediated endosomal escape of genetic cargo. Although PEGylation is a useful strategy to prolong the circulation time of nanoparticles in vivo by avoiding their rapid capture and clearance by undesired tissues and cells, such as macrophages, it also compromises endosomal release, known as the "PEG dilemma". It has been well documented that the use of polyethylene glycol (PEG) in order to create long-circulating nanoparticles, inhibits the endosomal escape of lipoplexes as well as polyplexes.[52−55] For lipoplexes this is expected as a hydrophilic PEG layer will prevent the intimate contact between lipoplex and endosome membrane, that is required for lipid mixing and, consequently, endosomal escape. However, PEGylation of polyplexes is not expected to influence the proton buffering capacity of the polymers. Therefore, since PEG prevents endosomal escape of polyplexes, it seems reasonable to suggest that in addition to the proton sponge effect an intimate contact between polymers and the endosomal membrane is required for endosomal destabilization and nucleic acid release into the cytosol. Indeed, protonation of polymers will -next to endosome swelling- also cause polymer swelling due to electrostatic repulsion between the protonated polymers. These protonated polymers will show enhanced electrostatic interaction with the endosomal membrane. Coarse-grained molecular dynamics simulations showed that adsorption of polymers to a membrane under tension, as induced by osmotic swelling and the increase in polymer size, may lower the critical stress that disrupts the membrane.[56] Therefore, cooperation of osmotic pressure and local membrane permeation by (expanded) polymer chains likely is responsible for efficient endosomal rupture.

By means of live cell imaging on a spinning disk confocal microscope, Rehman et al. showed the involvement of an endosomal burst in polyplex-mediated delivery of nucleic acids,

DOI: 10.1021/acs.accounts.9b00177
Acc. Chem. Res. XXXX, XXX, XXX−XXX

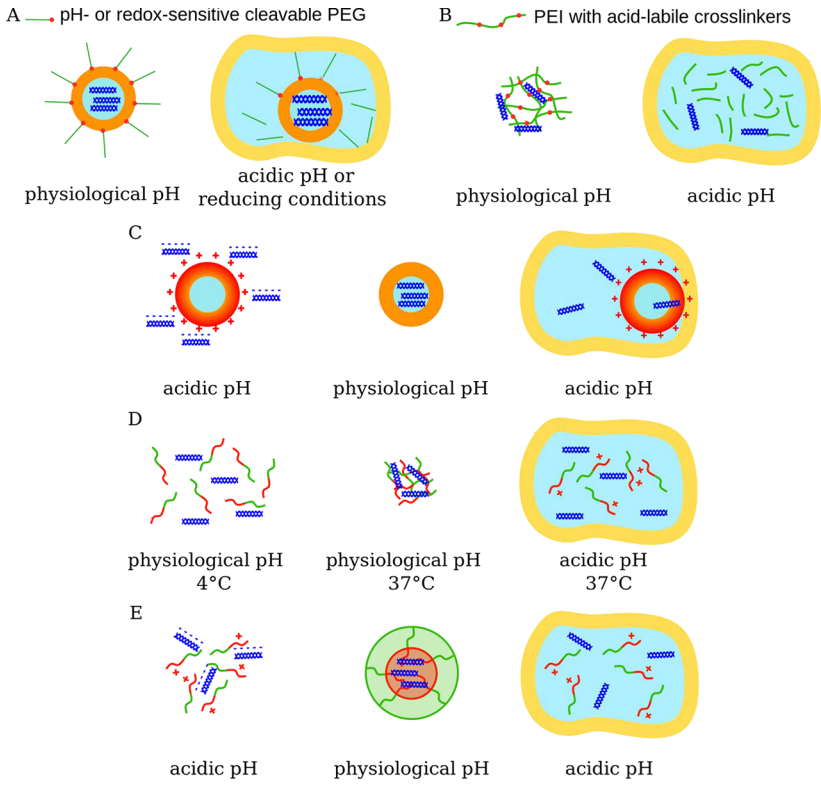

Figure 4. Stimuli-responsive nanomaterials for gene delivery. (A) Nanocarrier coated with PEG via an acid- or reduction-labile linker. Removal of the PEG coating occurs at acidic pH (in endosome) or in a reducing environment (in endosome?, cytosol, nucleus), respectively.[61,63,73] (B) Polyplex with pH-sensitive PEI. Low molecular weight PEI chains are linked via acid-labile bonds. Upon acidification (in endosome), the cross-linker is degraded. This approach combines the advantages of high molecular weight PEI (high transfection efficacy) and low molecular weight PEI (low cytotoxicity).[62] (C) Ionizable lipid. At acidic pH, the lipids are positively charged, inducing electrostatic interaction with negatively charged nucleic acids. At physiological pH, the lipid−nucleic acid complex has a near-neutral charge, promoting long blood circulation time. At acidic pH (in endosome), the lipids become positively charged and destabilize the endosomal membrane, leading to the release of nucleic acids in the cytosol.[64−67] (D) pH-Responsive thermogelling polymer. The polymer is water-soluble at 4 °C and forms a solid gel at 37 °C, at physiological pH. Encapsulation of nucleic acids occurs at the sol−gel transition. At acidic pH (in endosome), the pH-sensitive moiety (red) is protonated and the gel solubilizes, releasing its cargo.[69] (E) pH-Responsive block copolymer. The copolymer consists of a hydrophilic pH-insensitive polymer (green) and a polymer that is hydrophobic at neutral pH, whereas it becomes protonated and turns hydrophilic at a low pH (pH < pK_a).[71] Block copolymers are loaded with nucleic acids through a pH switch method. At endosomal pH, the copolymer turns hydrophilic, which will lead to complex dissociation.[70−72]

providing direct evidence for osmotic rupture of endosomes through the so-called proton sponge effect.[49] Specifically, incubation of cells with LPEI polyplexes, comprising fluorescently labeled polymers and ODNs, presented a burst release of the genetic cargo from typically only one or two endosomes per cell, with a complete discharge of the nucleic acid content from the endosome into the cytosol (Figure 2B). Interestingly, a seemingly vectorial release of genetic cargo from a polyplex-containing endosome was observed, hinting to the involvement of local destabilization of the endosomal membrane.[49] On the contrary, by means of super-resolution imaging (STORM) of fixed cells, intact cationic glycogen-siRNA complexes (glycoplexes) were shown to be released from endosomes, while glycoplex disassembly occurred in the cytosol (Figure 2C).[57] Because glycoplexes behave as rigid globular spheres, association of glycogen chains with the endosomal membrane likely is prevented. It was therefore concluded that endosome rupture by glycoplexes is exclusively determined by endosome swelling, i.e., without a role for permeation of the endosomal membrane by expanded polymers.[57] Whether local destabilization of the endosome membrane by positively charged (expanded) polymer (chains) facilitates the endosomal rupture induced by polyplexes remains unclear, and may be dependent on cell type, carrier type, and/or type of cargo. The combination of super resolution microscopy with live cell imaging will provide exciting opportunities to study nanocarrier−cell interactions, which is expected to give even more detailed information on the dynamics of endocytosis and cargo release from endosomes.[58]

5. STIMULI-RESPONSIVE MATERIALS FOR NANOCARRIER STABILITY IN CIRCULATION AND CONTROLLED RELEASE OF CARGO AT THE TARGET SITE

Over the past decades, numerous nanoparticle platforms have been investigated for drug delivery, including liposomes, polymeric nanoparticles, dendrimers, and inorganic/semi-inorganic nanoparticles. Although these materials (mostly polymers and lipids) account for >80% of the therapeutic nanoparticles that are in clinical use, there now is a tendency to develop stimuli-responsive nanoparticle platforms that are sensitive to specific signals and that may contribute to site-specific drug delivery.[59] Exogenous stimuli-responsive drug delivery systems respond to noninvasive physical triggers, including light, heat, ultrasound, and magnetic field, while

DOI: 10.1021/acs.accounts.9b00177
Acc. Chem. Res. XXXX, XXX, XXX−XXX

endogenous stimuli-responsive systems respond to specific biochemical conditions, such as a change in pH, redox state, or the presence of specific enzymes.[60] Distinct enveloped viruses that enter cells via endocytosis, exploit this pathway by a required pH-dependent change in one of the viral proteins (such as hemagglutinin (HA) protein of the influenza virus) that facilitates fusion between the viral envelope and the endosomal membrane, thereby causing endosomal escape of the viral RNA and/or DNA. Inspired by this strategy, a concept for applying pH-sensitive nanoparticles has been proposed, involving nanoparticles that are stable at physiological pH, but dissociate at mild acidic pH. For example, pH-sensitive nanocarriers can be prepared from lipids or polymers that contain acid-labile bonds that trigger particle dissociation and drug release upon a decrease in pH (Figure 4 A,B).[61−63] Such a decrease in pH occurs, e.g., in the interstitial fluid of tumors or within endosomes upon endosomal maturation. The use of acid-labile nanocarrier components may provide a potential solution for the "PEG dilemma", i.e., the prolongation of blood circulation time of gene vectors upon their pegylation that goes at the expense of their endosomal escape capacity. Chan et al. recently reported that PEGylated cationic liposomes containing a new acid-labile PEG-lipid were able to destabilize the endosomal membrane and induce efficient transfection.[63]

5.1. Ionizable Lipids in Lipid Nanoparticle (LNP) Systems for Gene Delivery

Recent development of lipids and polymers that display pH-induced structural changes aim for the generation of nanocarriers that are stable in the blood circulation thanks to their (near) neutral charge at physiological pH, for in vivo gene delivery purposes. A low pH-induced membrane-destabilizing activity provides those carriers with the necessary endosomolytic activity to release their genetic cargo into the cytoplasm. Cullis and co-workers developed lipid nanoparticles (LNPs) with ionizable aminolipids, e.g., 1,2-dioleoyl-3-dimethylammonium propane (DODAP) and heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA), that change to a cationic form under acidic conditions.[64,65] At acidic pH the aminolipids effectively complex (negatively charged) nucleic acids (DNA, RNA). When, after complex formation, the pH is neutralized (∼pH 7), the complexes exhibit a near-neutral surface charge, which prolongs their blood circulation time. Due to endosomal acidification during the endocytic processing of the complexes, the aminolipids again become cationic (Figure 4C). Close proximity between the LNP and the endosomal membrane allows for lipid mixing and the formation of ion pairs between the cationic lipids and anionic lipids within the endosome, which induces inverted nonbilayer conformations that exert a membrane-destabilizing effect. This will result in the release of the genetic cargo into the cytoplasm. Although the ionizable lipids DLin-KC2-DMA and DLin-KC4-DMA showed comparable pK_a values, and bilayer-to-hexagonal H_{II} transition temperatures (T_{BH}) when mixed with anionic lipids, they showed a >30-fold difference in in vivo transfection activity.[66] In this study, the T_{BH} was determined by differential scanning calorimetry of a suspension of ionizable lipids premixed with the anionic lipid DSPS. However, premixing of cationic (ionizable) lipids and anionic lipids may not properly reflect the situation in cells where the cationic lipids first need to gain access to the LNP−endosomal membrane interface. This property is likely influenced by the length and flexibility of the spacer between the charged lipid headgroup and the lipid tails, as DLin-KC2-DMA and DLin-KC4-DMA only differ in spacer group.[66] As previously mentioned, cell-based assays to quantify the endosomal escape of genetic cargo are important to confirm the endosomolytic potential of nanocarriers, as determined by physicochemical analyses, and are instrumental in screening gene delivery vectors for transfection activity. Recently, Sato et al. synthesized a new pH-sensitive cationic lipid YSK05 that was incorporated in LNPs for gene silencing. With their LNP formulation, that required prior complexation of siRNA with protamine for efficient loading with siRNA, they achieved in vitro (in HeLa cells) and in vivo (by direct tumor injection) siRNA-mediated gene silencing.[67] Likewise, pH-sensitive amino lipids have been successfully employed for DNA delivery.[68]

5.2. pH-Sensitive Block Copolymers for Gene Delivery

Next to pH-sensitive lipids, pH-sensitive block copolymers are investigated for drug delivery purposes. Recently, block copolymers with poly(*N*,*N*′-diethylaminoethyl methacrylate) (PDEAEM) were prepared to increase the hydrophilicity of the polymer and improve drug loading. These block copolymers formed hydrogels at body temperature, and drug release from these gels was influenced by the environmental pH (Figure 4D).[69] The diblock copolymer poly(2-methacryloyloxyethyl phosphorylcholine)-*b*-poly(2-(diisopropylamino)-ethyl methacrylate) (PMPC-PDPA) was successfully employed for siRNA delivery.[70] PDPA has a pK_a of 6.4. It is hydrophobic at neutral pH, whereas it becomes protonated and turns hydrophilic at a pH < 6.4.[71] Similar to LNPs containing ionizable aminolipids, PMPC-PDPA block copolymers could be loaded with siRNA at acidic pH. It is postulated that after their uptake by cells, the polymers become hydrophilic at endosomal pH, which will lead to complex dissociation (Figure 4E). This will lead to release of the genetic cargo, and at the same time generate osmotic stress within the endosomes, that in turn will lead to endosomal rupture and release of the genetic cargo in the cytoplasm. However, inclusion of the pore forming drug amphotericin B was required to obtain effective endosomal escape of siRNA when using another PDPA block copolymer for delivery, i.e., poly(2-(dimethylamino)ethyl methacrylate PDMA−PDPA,[72] indicating that here PDPA-generated osmotic stress was not sufficient for inducing endosomal disruption. Alternatively, dual responsive systems that combine, e.g., pH- and redox-responsiveness may improve endosomal destabilization. Zhu et al. made a pH/redox dual-responsive mPEG−PLA-PHis-ssOEI polyplex.[73] The structure change of this polyplex at low pH led to exposure of the redox cleavable disulfide bridges, promoting complex dissociation and endosome membrane destabilization. Of note, the N/P ratio turned out to be an important determinant for complex dissociation, because at high N/P ratio (10) enhanced nucleic acid binding due to protonation at low pH, counteracted the complex dissociation induced by the hydrophobic−hydrophilic switch of the polymer.[73]

6. CONCLUSIONS AND PERSPECTIVES

The exact parameters that would determine and/or predict the quality of a perfect drug/gene nanocarrier, ready for clinical use, remain largely enigmatic and difficult to define. Clearly, issues of concern are stability, high-efficiency, low-cytotoxicity, large payload, potency of production scale-up, economics, etc.

DOI: 10.1021/acs.accounts.9b00177
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Even though major efforts have been undertaken over the past decades to develop nanoscale materials for the delivery of (therapeutic) molecules, for disease diagnosis and treatment, the outcome has not yet met the expectations. As an example, of nonviral vectors only a few modified lipid- and polymer-based nanocarriers have entered the clinical trial phase, while viral vectors constitute approximately 70% of the clinical trials.[1,2] Though safety concerns partly restrict the application, low efficiency seems a major limitation for nonviral vectors so far. Many in vitro well-evaluated nonviral vectors fail to cross the biological barriers in vivo, therefore resulting in low efficiency. Improvement of physical-chemical and biological activity of nonviral vectors is therefore still urgently needed.

In nature, several pathogens and secreted toxins use specific strategies for crossing extracellular and intracellular biological barriers in order to enter host cells. The molecular mechanisms that are involved in the host cell invasion by pathogens have inspired the development of biomimetic nanocarriers for drug delivery. Various studies have estimated that optimized surface modification with pathogen-derived molecules improves selective delivery in the biological system. The development of biomimetic nanocarriers for drug/gene delivery is a rapidly emerging field, which exploits advantages of molecular mechanism used by pathogens. However, this concept is still in progress, and whether safety poses a risk in copying these strategies requires further studies. An increase in knowledge on how pathogens control host cell signaling and invasion, and advances in the synthesis of novel material may hold a promising and bright future for nanotechnology, especially the development of stimuli-responsive materials such as a sensitivity toward pH, temperature, redox, or magnetic field. These innovative materials should lead to a new field of site-specific and/or programmable drug delivery.

The use of new tools for the exploration of nanocarrier−cell interactions in vitro and in vivo, including refined methods to study endocytosis, and advances in fluorescence labeling strategies and high resolution microscopy techniques will be particularly helpful in moving the nanomedicine field forward.

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Notes

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Cuifeng Wang did her PhD in the lab of Dr. Inge Zuhorn on pathogen-inspired gene delivery systems, being a recipient of a Chinese Research Council (CSC) fellowship. Her current work at the School of Pharmaceutical Sciences at Sun Yat-Sen University (Guangzhou, China) in the lab of Prof. Min Feng focuses on the development of drug delivery systems for cancer therapy.

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ACKNOWLEDGMENTS

I.S.Z. and I.M.S.D. are supported by the Dutch Technology Foundation TTW, which is part of The Netherlands Organisation for Scientific Research (NWO), and which is partly funded by the Ministry of Economic Affairs.

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DOI: 10.1021/acs.accounts.9b00177
Acc. Chem. Res. XXXX, XXX, XXX−XXX